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CRYSTALLIZATION OF CALCIUM OXALATE AND CALCIUM PHOSPHATE AT SUPERSATURATION LEVELS CORRESPONDING TO THOSE IN DIFFERENT PARTS OF THE NEPHRON

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Abstract

The risk of crystallization in solutions, with a composition corresponding to that of urine in various parts of the nephron, was assessed by studying urine samples containing standardized increments of calcium and oxalate concentrations. The experiments were carried out in salt solutions with and without dialysed urine and the appearance of crystals was detected and measured with a Coulter counter. With increased concentrations of calcium, crystals of calcium phosphate were predominantly formed in solutions with a composition corresponding to that in the proximal and distal tubuli, whereas, calcium oxalate was the primary crystallization product in solutions with a composition corresponding to the collecting duct. These conclusions were based on calculations of ion-activity products of calcium oxalate, hydroxyapatite, and calcium hydrogen phosphate, at the first appearance of crystals; studies on crystal morphology with scanning electron microscopy, and precipitation of [^{14}C]-oxalate following addition of calcium. The ion-activity products of calcium oxalate at the first appearance of crystals following addition of oxalate to solutions with a composition corresponding to the collecting duct were significantly lower in the presence of dialysed urine. This might reflect a promoting effect of some macromolecules on the nucleation of calcium oxalate. Dialysed urine in these samples also had a pronounced influence on the development of the crystals by markedly reducing the mean crystal volume during the first hour following the formation of 100 crystals with a diameter between 3.5 and 5 μm. Such an effect was not observed in those solutions in which calcium phosphate crystals had formed.

Key Words: Crystallization, calcium oxalate, calcium phosphate, renal tubuli, collecting duct, promotion, inhibition, urine, macromolecules.

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Introduction

The mechanisms behind the formation of calcium renal stones are incompletely understood, particularly concerning the initial crystallization events. With sensitive stone analyses, it has become apparent that a large proportion of these stones are composed of mixtures of calcium oxalate and calcium phosphate (Prien, 1955; Pak *et al.*, 1971; Chambers *et al.*, 1972; Larsson *et al.*, 1984a, b; Ohkawa *et al.*, 1992). A comparison of urine composition in patients with different stones also showed that the excretion of oxalate obviously was increased in patients with pure calcium oxalate stones, this was usually not the case in patients with mixed stones. Stones containing both calcium oxalate and calcium phosphate appeared to be associated with other risk factors than stones with only calcium oxalate (Peacock *et al.*, 1979; Öhman *et al.*, 1992).

It is reasonable to assume that the formation of renal stones starts as a crystallization at the nephron level, probably due to an interaction of the driving force of the supersaturated urine and the cells lining the tubuli or the collecting ducts.

When we estimated the supersaturation levels of calcium salts in different parts of the nephron based upon the information in the literature (Rector, 1973; Koushanpour, 1976; Robertson, 1976; Tiselius *et al.*, 1978; Tiselius, 1982; Simpson, 1983; Kasidas and Rose, 1986), the data indicated that the risk of calcium phosphate crystallization might exceed that of calcium oxalate. This appeared to be the case particularly for a urine composition corresponding to that in proximal tubuli, loop of Henle, and distal tubuli. High supersaturation levels and a risk of precipitation of both calcium phosphate and calcium carbonate in the tubular system, particularly the loop of Henle, also has been reported recently (Coe and Parks, 1990; Deganello *et al.*, 1990).

The present study was undertaken as an attempt to increase our understanding of the formation of crystals in association with increased concentrations of calcium and oxalate in a urine with a composition otherwise similar to that in the parts of the nephron.

Table 1. Composition of salt solutions corresponding to that in urine from different parts of the nephron.

		Proximal tubuli (PT)	Distal tubuli prox part (DTp)	Distal tubuli dist part (DTd)	Collecting ducts (CD)
Calcium	mmol/l	2.78	1.32	1.04	4.50
Magnesium	mmol/l	0.09	0.12	0.41	3.85
Phosphate	mmol/l	1.00	1.25	4.17	32.3
Oxalate	mmol/l	0.010	0.013	0.04	0.32
Citrate	mmol/l	0.08	0.10	0.35	3.21
Sodium	mmol/l	139	82	96	106
Potassium	mmol/l	3.2	0.95	22.5	63.7
Sulfate	mmol/l	3.3	4.2	13.8	20.8
pH		6.75	6.45	6.45	5.75

Methods

Crystallization systems

Several salt solutions were prepared in accordance with the assumed ion composition of urine in the proximal tubuli (PT), the proximal part of the distal tubuli (DTp), the distal part of the distal tubuli (DTd), and the collecting ducts (CD). The compositions of these solutions, which is summarized in Table 1, were based on data in the literature concerning ion-concentrations in the nephron, information on the renal handling of the constituents, as well as their plasma concentrations and excretion in urine. For these calculations, we assumed that the concentrations of the various urine variables in the glomerular filtrate were as follows: calcium: 1.5 mmol/l; oxalate: 2 μmol/l; magnesium: 0.54 mmol/l; phosphate: 1.35 mmol/l; sodium: 135 mmol/l; potassium: 3.8 mmol/l; citrate: 0.07 mmol/l; sulfate: 1.4 mmol/l; and pH: 7.4 (Koushanpour, 1976; Robertson, 1976; Cohen and Barac-Nieto, 1983; Kasidas and Rose, 1986; Simpson, 1983). An average 24-hour volume of the glomerular filtrate of 180 liters was expected to be reduced to 36 liters at the end of PT; 28.8 liters at the end of Henle's loop; 8.6 liters at the end of DT; and 1.44 liters at the end of CD (Koushanpour, 1976). The concentrations of calcium were based on a reabsorption of 63% in PT, 23% in DTp, 11% in DTd, and 1% in CD (Lassiter *et al.*, 1963; Robertson, 1976). The reabsorption of magnesium, which mainly occurs in the proximal part of the nephron (Walser, 1983) was roughly assumed to be 93% in PT and 3.5% in DT.

The concentration of oxalate was considered to be affected only by the volume reduction of urine and no attention was paid to any secretion or passive reabsorption that might occur. The reabsorption of citrate varies considerably with the acid base balance, but has been reported to be around 75% under normal circumstances (Robertson, 1976). This value was used as an estimate of the reabsorption of citrate in PT. The tubular reabsorptive capacities for phosphate (Koushanpour, 1976) and sulfate in PT (Koushanpour, 1976; Lingeman *et al.*, 1989) were assumed to be 140 and 80-90 mmol per 24 hours, respectively. Eighty per cent of filtered sodium is reabsorbed in PT. Of the total filtered sodium, 9.4% has been reported to remain in PT, 3.3% in DT, and 0.7% in CD (Koushanpour, 1976). The reabsorption of potassium in PT was considered to be 84%, with 3.8% of filtered potassium remaining in DTp and 27% in DTd, attributable to an inflow of potassium. At the end of CD, 91.4% of filtered potassium was assumed to be reabsorbed (Koushanpour, 1976).

The pH in the samples was chosen according to results reported by Rector (1983) with 6.7-6.8 in PT; 6.4-6.5 in DT; and 5.6-6.0 in CD.

These solutions were used in the crystallization experiments either with or without the addition of dialysed urine. In order to avoid any risk of precipitation of ammonium salts, we added no ammonium ions to the solutions, despite the normal presence of this ion both in the distal tubuli and the collecting ducts. Because of technical difficulties in preparing a CD solution according to these calculations, we used a composition of the CD sample described for urine by Burns and Finlayson (1980).

The amount of dialysed urine added to the samples was roughly calculated from the degree of dilution at different nephron levels in comparison with the final urine. Dialysed urine was added to the PT and the DTp samples to give a 10% concentration of urine. The DTd samples were analyzed with a 20% urine concentration, while the CD sample was prepared by dissolving the necessary salts in the dialysed urine.

Preparation of dialysed urine

A pooled sample of urine from normal subjects was used as a source of macromolecules. This urine had been prepared according to our routine for direct determination of the risk of crystallization (Tiselius, 1985). This urine had been collected between 10 PM and 6 AM in bottles containing 15 ml of 3 mmol/l sodium azide. Each sample was checked for the absence of bacteria,

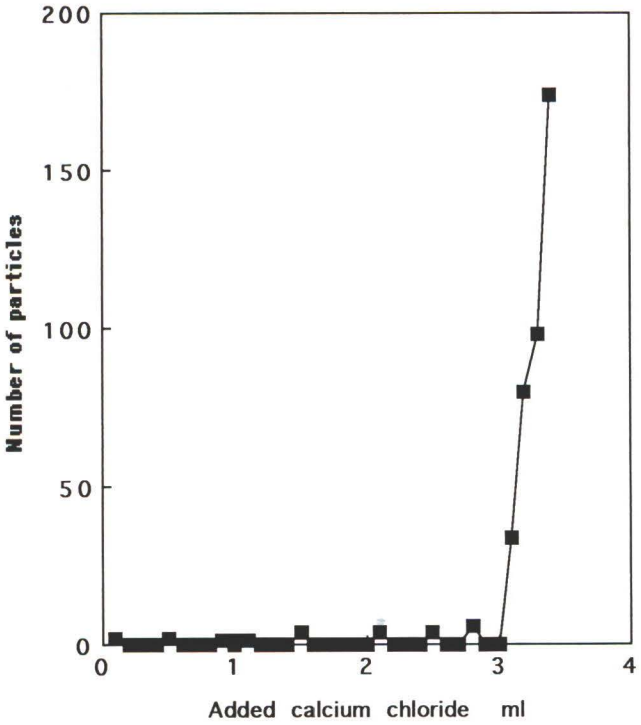

Figure 1. A typical result of the crystal formation in a sample following standardized addition of calcium. The recording of crystals was made in the size range 3.5 to 5 μm in a Coulter counter 40 seconds after each addition of 100 μl of a 1 mol/l calcium chloride solution.

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protein, and glucose, after which pH was adjusted to 5.8. The urine was subsequently centrifuged at 2800 rpm for 30 minutes. The supernatants were decanted and saved, and the sediments were combined in one tube. Following repeated centrifugation of the sediment fraction, this supernatant was combined with the previously collected supernatants. The crystals in the sediment were dissolved by addition of 1 mmol/l hydrochloric acid. After centrifugation and neutralization, this supernatant was combined with the other urine.

The undissolved material in the tube was discarded. The pH of the urine was now readjusted to 5.8. One hundred milliliter aliquots of the pooled urine were then transferred to Spectrapore No. 3 dialysis tubings with an exclusion limit of 3500 Da (Spectrum Medical Industries, Inc., Houston, Texas). Each tubing was placed in 1000 ml of deionized water with magnetic stirring at room temperature. The water was exchanged eight times, once every hour. During the following 15 hours, still under continuous stirring, the samples were kept in deionized water at +4°C, after which the deionized water was replaced by Milli-Q filtered water (Millipore, S.A, Molsheim, France), exchanged six times, once every hour. The samples were subsequently filled up to the original volume with water and stored at -20°C until used in the experiments.

Determination of crystallization following addition of calcium chloride

One hundred milliliter samples of the salt solutions, with or without dialysed urine, were passed through 0.22 μm Millipore filters. A solution of calcium chloride was added in 100 μl fractions to the PT, DTd, and CD samples, and in 200 μl fractions to the DTp samples. Calcium chloride was added every minute and the number of crystals recorded 40 seconds later with a Coulter counter (Model Z_B) equipped with channelyser (Coulter Electronics Ltd, Luton, U.K.) using a 100 μm capillary tube. Only those crystals were considered that appeared in the size interval of 3.5 to 5.0 μm, and the addition of calcium was stopped when 100 crystals had been recorded in a volume of 50 μl. The pH was maintained by repeated addition of small aliquots of 0.05 mol/l sodium hydroxide.

Due to differences in the crystallization propensity, two concentrations of calcium chloride were used in the experiments. A 1.0 mol/l calcium chloride solution was used for PT, DTp, and DTd samples, while, a 0.7 mol/l solution was used in the experiments with CD samples.

A typical crystallization experiment is shown in Figure 1. The concentrations of the constituents of the samples at the first appearance of crystals, as well as at the formation of 100 crystals were recorded. Corrections were made for changes in the volume as a result of crystal counting. These data were subsequently used for calculation of the ion-activity products of calcium oxalate (AP_{CaOx}); hydroxyapatite (AP_{HAP}); and calcium hydrogen phosphate, brushite (AP_{Bru}); by means of a computerized iterative approximation with the EQUIL2 program (Werness *et al.*, 1985). The risk of crystallization was also expressed as the increment in calcium concentration necessary for formation of crystals in the size range 3.5 to 5 μm.

Determination of crystallization following addition of sodium oxalate

One hundred milliliter samples of salt solutions, with or without dialysed urine, were passed through 0.22 μm Millipore filters. A solution of 0.04 mol/l sodium oxalate was added in 100 μl fractions to all samples. Sodium oxalate was added every minute and the number of crystals recorded 40 seconds later as described above for the calcium experiment (Tiselius, 1985). The concentrations of the constituents were recorded in a similar way, both at the first appearance of crystals and at the formation of 100 crystals in the size range 3.5 to 5 μm. The pH was checked before and after crystallization and remained constant.

Determination of mean crystal volumes

As soon as 100 crystals had been recorded following addition of the calcium, the samples were kept under magnetic stirring during the following 60 minutes. Aliquots were drawn from all samples after 15 and 60 minutes for analysis of crystal size distribution in a Coulter Multisizer with a 100 μm capillary tube. The number

and volume of the crystals were determined in the size interval between 3.5 and 45 μm. The mean crystal volume (MCV) was obtained by dividing the total volume (μm^3) by the total number of crystals. We also calculated MCV-quotients: MCV_{60}/ MCV_{15}, to express the changes in crystal volume that occurred during the observation period.

Microscopic examination of the crystals

Aliquots for examination of the crystal material after addition of calcium chloride were obtained from samples treated in the same standardized way as described above. Samples were drawn just before the expected appearance of 3.5 to 5 μm crystals in the solution. These samples were isolated for scanning electron microscopy (SEM) by passing them through polycarbonate membrane filters with a pore size of 0.2 μm (Poretics corp, Livermore, CA, USA). The filters were rinsed with air, dried at room temperature, and mounted with double stick tape on metallic stubs. The crystals were then covered by a 10 nm layer of metallic platinum in a twin electron beam gun sputter coating unit (Edwards Model 3AM, Crawley, Sussex, U.K.). The crystals were examined in a JEOL JSM-840 SEM (Tokyo, Japan).

Isotope-studies of crystallization

In this experiment calcium chloride was added to PT, DTp, DTd, and CD samples containing 10 μCi of [^{14}C]-oxalate per ml. The [^{14}C]-oxalic acid solution had a specific radioactivity of 109 μCi/μmol (Amersham, Buckinghamshire, U.K.). After each addition of calcium chloride to 100 ml of the experimental solution, aliquots of one milliliter were drawn for assessing the amount of isotope remaining in solution. After passage of the sample through a 0.22 μm Millipore filter, 0.5 ml was immediately added to 10 ml of scintillation fluid (Maxi-fluor, J T Baker BV, Deventer, Netherlands). The isotope concentration was measured in a liquid scintillation spectrometer (LKB Wallac 1217, Turku, Finland). Quench correction was performed by means of an internal standard.

Calculations of ion-activities

For calculation of the ion-activity product of HAP we used the structure $Ca_5(PO_4)_3(OH)$.

Statistics

Mann-Whitney U test were used for sample comparisons.

Results

The ion-activity products of calcium oxalate (AP_{CaOx}) at the first appearance of crystals in salt solutions, with and without dialysed urine, following addition of calcium, are presented in Table 2. The saturation with respect to CaOx was very low in all solutions except in the CD samples. With a thermodynamic solubility product for CaOx of 0.23 to 0.25 x 10^{-8} M^2 (Pak *et al.*, 1975; Tomazic and Nancollas, 1979), it is evident that the AP_{CaOx} values in both PT and DTp samples were below that level. These solutions were thus undersaturated with CaOx at the time of crystal formation, and the only type of crystal observed in PT and DTp samples was calcium phosphate. Although the AP_{CaOx} in DTd samples was above the solubility product, this supersaturation is certainly too low to be associated with a nucleation of CaOx. In contrast, the CD samples were highly supersaturated with CaOx.

The AP_{HAP} values corresponding to the first appearance of crystals as well as when 100 crystals with diameters between 3.5 and 5 μm were recorded, are given in Table 3. The lowest supersaturation levels associated with the formation of calcium phosphate crystals was observed in the DTd samples. The formation product of HAP is unknown, but the AP_{HAP} values in all samples were considerably above the solubility product for HAP, which is reported to be approximately 2.4 x 10^{-59} M^9 (Koutsoukos and Nancollas, 1981).

The pH levels in the nephron are consistent with precipitation of calcium hydrogen phosphate and in as much as some crystals observed in the PT, DTp, DTd, and CD samples, 60 minutes after the end point in the calcium experiments, had a brushite-like appearance, we also calculated the AP_{Bru} in the different samples (Table 4). Although the supersaturation with this salt was below the reported formation product [2 x 10^{-7} $(mol/l)^2$], the values exceeded the solubility product of 0.9 to 1.3 x 10^{-7} (Koutsoukos and Nancollas, 1981; Nancollas, 1982) in PT, DTp, and DTd samples. The AP_{Bru} at the first appearance of crystals were significantly lower in DTp-samples with dialysed urine ($p < 0.01$) and higher in CD-samples with dialysed urine ($p < 0.01$).

The ion-activity products of CaOx, HAP and brushite at the crystallization that followed increased concentrations of oxalate are shown in Tables 5, 6 and 7. In all solutions, except in the CD samples, the AP_{CaOx} required for oxalate induced crystallization was significantly higher ($p < 0.01$) than that associated with crystallization following addition of calcium. The AP_{HAP} and AP_{Bru} levels recorded at crystallization following addition of oxalate were significantly lower ($p < 0.01$) than those observed following addition of calcium.

The risk of crystallization expressed as the relative increment in the concentrations of calcium and oxalate, required for the formation of crystals in the individual solutions, is shown in Table 8. It is evident that a great risk of crystallization following addition of calcium occurred in PT, DTd, and CD samples, while CD samples showed the greatest risk following the addition of oxalate. In the PT and DT samples, a much higher relative increment in the concentration of oxalate than of calcium was necessary to induce crystal nucleation.

The concentration of [^{14}C]-oxalate remaining in solution at several concentrations of calcium is shown in Figure 2. There was evidently a marked precipitation of oxalate only in the CD samples, with a consumption of isotope in PT, DTp, and DTd samples, with or without urine, of less than 5%. There was, however, a marked

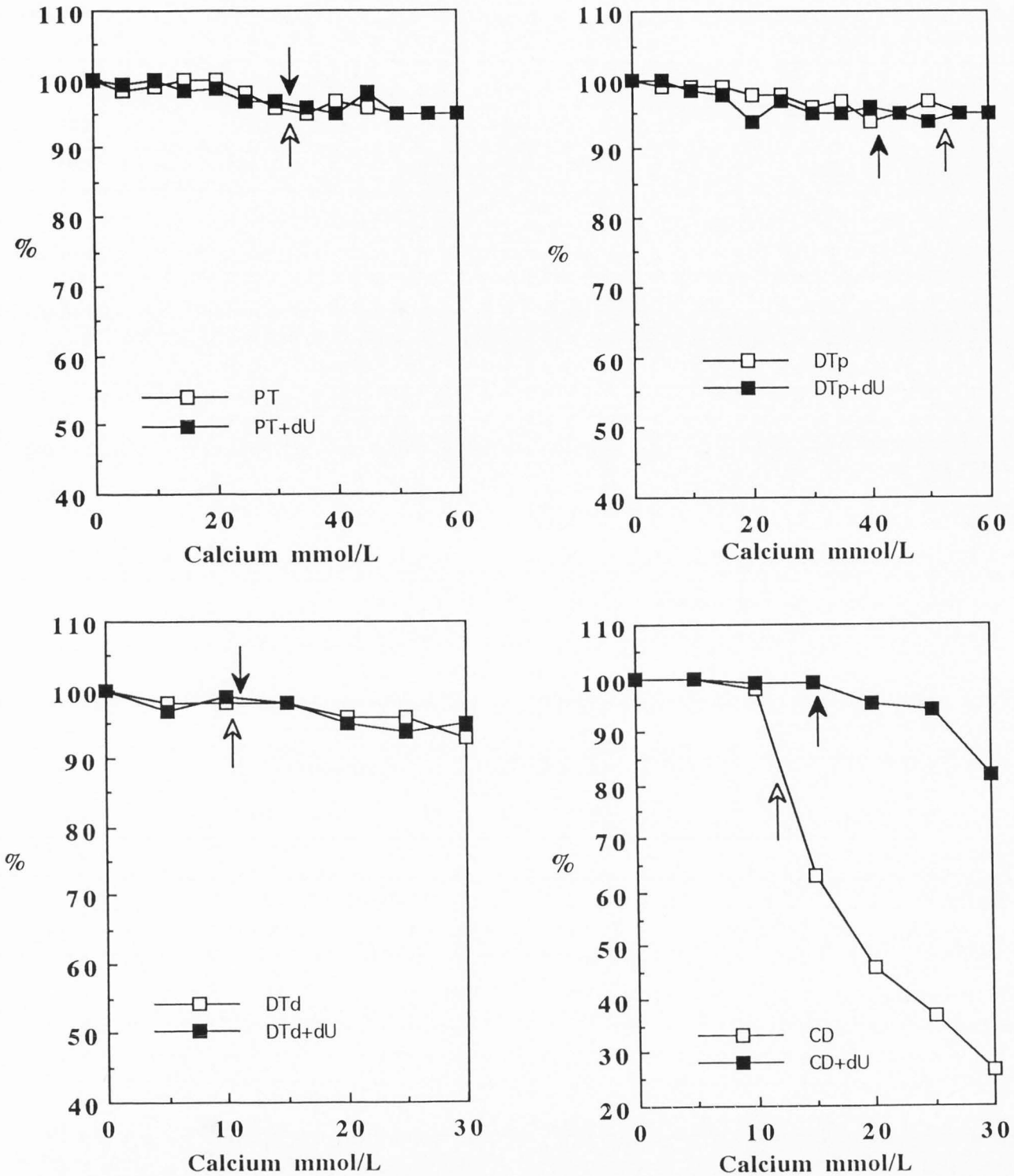

Figure 2. Per cent of [^{14}C]-oxalate remaining in solution following addition of calcium to solutions without and with dialysed urine. The arrows point to calcium concentrations where crystals first appeared in the Coulter counter experiments.

Table 2. Ion-activity products of calcium oxalate (AP_{CaOx}) at different points of crystallization in the experiments in which *calcium* was added.

$10^8 \times M^2$	Samples			
	PT	DTp	DTd	CD
Before calcium addition	0.103	0.102	0.154	1.95
Mean (Standard deviation, SD)		*Without dialysed urine*		
At first appearance of crystals in the size range 3.5-5 μm	0.11 (0.004)^m	0.10 (0.01)^m	0.63 (0.01)^m	4.15 (0.06)
At 100 crystals in the size range 3.5-5 μm	0.09 (0.005)^m	0.09 (0.01)^m	0.63 (0.10)^m	4.25 (0.02)
	n = 10	n = 10	n = 10	n = 11
		With dialysed urine		
At first appearance of crystals in the size range 3.5-5 μm	0.11 (0.006)^m	0.12 (0.08)^m	0.63 (0.14)^m	4.22 (0.06)
At 100 crystals in the size range 3.5-5 μm	0.09 (0.003)^m	0.10 (0.01)^m	0.62 (0.15)^m	4.18 (0.07)
	n = 10	n = 10	n = 10	n = 9

Compared to samples following addition of oxalate: $^m p < 0.01$.

Table 3. Ion-activity products of hydroxyapatite (AP_{HAP}) at different points of crystallization in the experiments in which *calcium* was added.

M^9	Samples			
	PT	DTp	DTd	CD
Before calcium addition	3.40×10^{-47}	2.29×10^{-50}	7.85×10^{-50}	3.66×10^{-49}
Mean (SD)		*Without dialysed urine*		
At first appearance of crystals in the size range 3.5-5 μm	2.17×10^{-42} (1.36×10^{-42})^m	0.86×10^{-42} (0.43×10^{-42})^m	2.08×10^{-44} (1.87×10^{-44})^m	0.61×10^{-45} (0.22×10^{-45})^m
At 100 crystals in the size range 3.5-5 μm	6.56×10^{-42} (1.78×10^{-42})^m	1.37×10^{-42} (0.70×10^{-42})^m	6.00×10^{-44} ($4.06\ 10^{-44}$)^m	2.11×10^{-45} (0.51×10^{-45})^m
	n = 10	n = 10	n = 10	n = 11
		With dialysed urine		
At first appearance of crystals in the size range 3.5-5 μm	2.58×10^{-42} (1.06×10^{-42})^m	0.29×10^{-42} (0.13×10-42)^{m,b}	3.06×10^{-44} (2.75×10^{-44})^m	1.97×10^{-45} (0.24×10^{-45})^m
At 100 crystals in the size range 3.5-5 μm	7.93×10^{-42} (1.38×10^{-42})^m	0.87×10^{-42} (0.26×10^{-42})^{m,b}	10.8×10^{-44} (7.52×10^{-44})^m	7.22×10^{-45} (0.47×10^{-45})^m
	n = 10	n = 10	n = 10	n = 9

Compared with samples without dialysed urine: $^b p < 0.01$;
Compared with samples following addition of oxalate: $^m p < 0.01$

Table 4. Ion-activity products of calcium hydrogen phosphate (AP_{Bru}) at different points of crystallization in the experiments in which *calcium* was added.

$10^7 \times M^2$	Samples			
	PT	DTp	DTd	CD
Before calcium addition	0.87	0.46	0.76	4.21
Mean (SD)		*Without dialysed urine*		
At first appearance of crystals in the size range 3.5-5 μm	5.58 (0.16)^{m}	7.23 (0.31)^{m}	7.50 (1.17)^{m}	17.1 (1.10)^{m}
At 100 crystals in the size range 3.5-5 μm	6.20 (0.18)^{m}	7.76 (0.24)^{m}	9.39 (0.82)^{m}	21.6 (1.10)^{m}
	n = 10	n = 10	n = 10	n = 11
		With dialysed urine		
At first appearance of crystals in the size range 3.5-5 μm	5.53 (0.26)^{m}	6.52 (0.27)^{m,a}	7.99 (1.56)^{m}	20.7 (0.32)^{m,a}
At 100 crystals in the size range 3.5-5 μm	6.28 (0.13)^{m}	7.28 (0.19)^{m,a}	10.43 (1.06)^{m,a}	26.1 (0.40)^{m,a}
	n = 10	n = 10	n = 10	n = 9

Compared with samples without dialysed urine: $^{a}p < 0.05$; $^{b}p < 0.01$
Compared with samples following addition of oxalate: $^{m}p < 0.01$

Table 5. Ion-activity products of calcium oxalate (AP_{CaOx}) at different points of crystallization in the experiments in which *oxalate* was added.

$10^8 \times M^2$	Samples			
	PT	DTp	DTd	CD
Before oxalate addition	0.103	0.102	0.154	1.95
Mean (SD)		*Without dialysed urine*		
At first appearance of crystals in the size range 3.5-5 μm	4.94 (1.05)^{m}	4.38 (0.90)^{m}	4.05 (0.81)^{m}	5.30 (0.07)
At 100 crystals in the size range 3.5-5 μm	6.49 (0.27)^{m}	4.97 (0.78)^{m}	4.51 (0.71)^{m}	5.70 (0.19)
	n = 10	n = 10	n = 10	n = 10
		With dialysed urine		
At first appearance of crystals in the size range 3.5-5 μm	3.97 (0.17)^{m}	4.85 (0.94)^{m}	3.87 (0.85)^{m}	5.01 (0.23)^{a}
At 100 crystals in the size range 3.5-5 μm	5.20 (1.03)^{m,a}	5.65 (0.86)^{m}	4.38 (0.84)^{m}	5.62 (0.41)
	n = 10	n = 10	n = 10	n = 10

Compared to samples without dialysed urine: $^{a}p < 0.05$;
Compared to samples following addition of calcium: $^{m}p < 0.01$.

Table 6. Ion-activity products of hydroxyapatite (AP_{HAP}) at different points of crystallization in the experiments in which *oxalate* was added.

M^9	Samples			
	PT	DTp	DTd	CD
Before oxalate addition	3.40×10^{-47}	2.29×10^{-50}	7.85×10^{-50}	3.66×10^{-49}
Mean (SD)		*Without dialysed urine*		
At first appearance of crystals in the size range 3.5-5 μm	2.46×10^{-47} $(0.18 \times 10^{-47})^m$	1.33×10^{-50} $(0.16 \times 10^{-50})^m$	4.16×10^{-50} $(0.61 \times 10^{-50})^m$	3.19×10^{-49} $(0.08 \times 10^{-49})^m$
At 100 crystals in the size range 3.5-5 μm	2.21×10^{-47} $(0.04 \times 10^{-47})^m$	1.23×10^{-50} $(0.13 \times 10^{-50})^m$	3.83×10^{-50} $(0.50 \times 10^{-50})^m$	3.12×10^{-49} $(0.03\ 10^{-49})^m$
	n = 10	n = 10	n = 10	n = 10
		With dialysed urine		
At first appearance of crystals in the size range 3.5-5 μm	2.63×10^{-47} $(0.03 \times 10^{-47})^m$	1.25×10^{-50} $(0.16 \times 10^{-50})^m$	4.30×10^{-50} $(0.66 \times 10^{-50})^m$	3.23×10^{-49} $(0.04 \times 10^{-49})^m$
At 100 crystals in the size range 3.5-5 μm	2.42×10^{-47} $(0.17 \times 10^{-47})^m$	1.12×10^{-50} $(0.13 \times 10^{-50})^m$	3.93×10^{-50} $(0.59 \times 10^{-50})^m$	3.13×10^{-49} $(0.07 \times 10^{-49})^m$
	n = 10	n = 10	n = 10	n = 10

Compared to samples following addition of calcium: $^mp < 0.01$.

Table 7. Ion-activity products of calcium hydrogen phosphate (AP_{Bru}) at different points of crystallization in the experiments in which *oxalate* was added.

$10^7 \times M^2$	Samples			
	PT	DTp	DTd	CD
Before oxalate addition	0.87	0.46	0.76	4.21
Mean (SD)		*Without dialysed urine*		
At first appearance of crystals in the size range 3.5-5 μm	0.82 $(0.01)^m$	0.42 $(0.01)^m$	0.66 $(0.02)^m$	4.07 $(0.02)^m$
At 100 crystals in the size range 3.5-5 μm	0.80 $(0.003)^m$	0.41 $(0.01)^m$	0.65 $(0.02)^m$	4.05 $(0.01)^m$
	n = 10	n = 10	n = 10	n = 10
		With dialysed urine		
At first appearance of crystals in the size range 3.5-5 μm	0.83 $(0.002)^m$	0.41 $(0.01)^m$	0.67 $(0.02)^m$	4.08 $(0.01)^m$
At 100 crystals in the size range 3.5-5 μm	0.82 (0 001)^m	0.40 $(0.01)^m$	0.66 $(0.02)^m$	4.06 $(0.02)^m$
	n = 10	n = 10	n = 10	n = 10

Compared to samples following addition of calcium: $^mp < 0.01$.

Table 8. Increments in the concentrations of calcium (Δ calcium) and oxalate (Δ oxalate) necessary for detection of crystals in the size range 3.5 to 5 μm in samples without and with (dU) dialysed urine. The relative increment was expressed as the quotient between the Δ calcium and the initial calcium concentration and between Δ oxalate and the initial oxalate concentration, respectively.

	Calcium addition		Oxalate addition	
	Δ calcium mmol/l	Relative increment	Δ oxalate mmol/l	Relative increment
PT	30.3 (2.0)	10.9	0.49 (0.1)	49.0
DTp	50.5 (5.8)	38.2	0.60 (0.14)	45.9
DTd	9.8 (2.0)	9.4	1.15 (0.26)	28.8
CD	7.5 (1.1)	1.7	0.57 (0.01)	1.8
PT + dU	29.8 (3.1)	10.7	0.39 (0.02)	38.7
DTp + dU	39.3 (3.6)	29.8	0.67 (0.14)	51.3
DTd + dU	10.7 (2.7)	10.3	1.08 (0.27)	27.0
CD + dU	11.0 (3.1)	2.4	0.52 (0.04)	1.6

difference in the crystallization of CaOx when the CD samples, with and without dialysed urine, were compared, with a much slower precipitation of oxalate in the presence of urine.

Scanning electron microscopy of the precipitate close to the first appearance of crystals in the size-range 3.5 to 5 μm showed that calcium phosphate, was probably the only early material in PT, DTp and DTd samples, following addition of calcium (Figures 3 and 4). In CD samples, with and without dialysed urine, CaOx was the only early crystal phase. When DTd and CD samples were further supersaturated by addition of calcium until 100 crystals in the size range 3.5 to 5 μm had formed, brushite crystals were frequently observed after 60 minutes.

The MCV following addition of calcium (Table 9) increased between 15 and 60 minutes with a factor of 1.6 to 5.5 in PT, DTp and DTd samples without dialysed urine. The corresponding figures in samples containing urine were 1.2 to 2.5. However, a most pronounced difference was recorded in the CD experiments, in which the MCV markedly increased in the absence of dialysed urine 36.1 compared with 1.04.

Table 9. Mean (SD) of the mean crystal volumes (MCV) and MCV-quotients at different times after formation of 100 crystals with a diameter between 3.5 and 5 μm following increment of the calcium concentration in solutions without and with dialysed urine.

	MCV 15 min μm^3	MCV 60 min μm^3	MCV60/ MCV15
PT	92.6 (22.4)	192.1 (56.6)	2.14 (0.72)
DTp	62.6 (13.6)	351.2 (306.1)	5.51 (4.82)
DTd	71.3 (38.5)	93.3 (30.9)	1.57 (0.85)
CD	65.2 (10.5)	2161 (1405)	36.1 (24.9)[a]
PT + dU	56.5 (11.2)	110 (20.3)	2.01 (0.54)
DTp + dU	63.4 (11.6)	158 (56.7)	2.49 (0.70)
DTd + dU	91.8 (47.7)	93.3 (52.1)	1.24 (0.89)
CD + dU	72.8 (39)	76.3 (22.3)	1.04 (0.13)

[a] $p < 0.02$ compared to samples with dialysed urine.

Discussion

It is reasonable to assume that renal stones composed of calcium salts start by a heterogeneous nucleation in a metastably supersaturated urine. Such a crystallization can theoretically be induced by free particles in the solution or by the surfaces of cells lining the collecting system, with or without a contribution of macromolecules (Vermeulen and Lyon, 1968; Finlayson and Reid, 1978; Finlayson, 1979; Rushton *et al.*, 1981; Hering *et al.*, 1989). The most frequent constituent of renal calcium stones is calcium oxalate and a recent analysis of the composition of non-infection calcium stones among our own patients revealed that whereas, 99% of calcium stones contained calcium oxalate, as many as 74% were mixtures of calcium oxalate and calcium phosphate (unpublished results). Information on the early steps of the formation of mixed calcium oxalate/calcium phosphate stones is insufficient due to experimental difficulties, and there is no agreement on whether calcium phosphate (Pak, 1969; Zarembski and Grieve, 1976; Baumann, 1985; Lanzalaco *et al.*, 1988; Achilles *et al.*, 1992) or calcium oxalate (Lonsdale, 1968; Finlayson, 1974; Sutor *et al.*, 1974; Hodgkinson, 1977; Hartung and Leskovar, 1978) constitutes the primary nucleus.

Methodological remarks

The composition of the solutions was chosen to correspond to an average composition of the urine in the respective parts of the nephron. This is undoubtedly an over-simplification because the composition of urine is

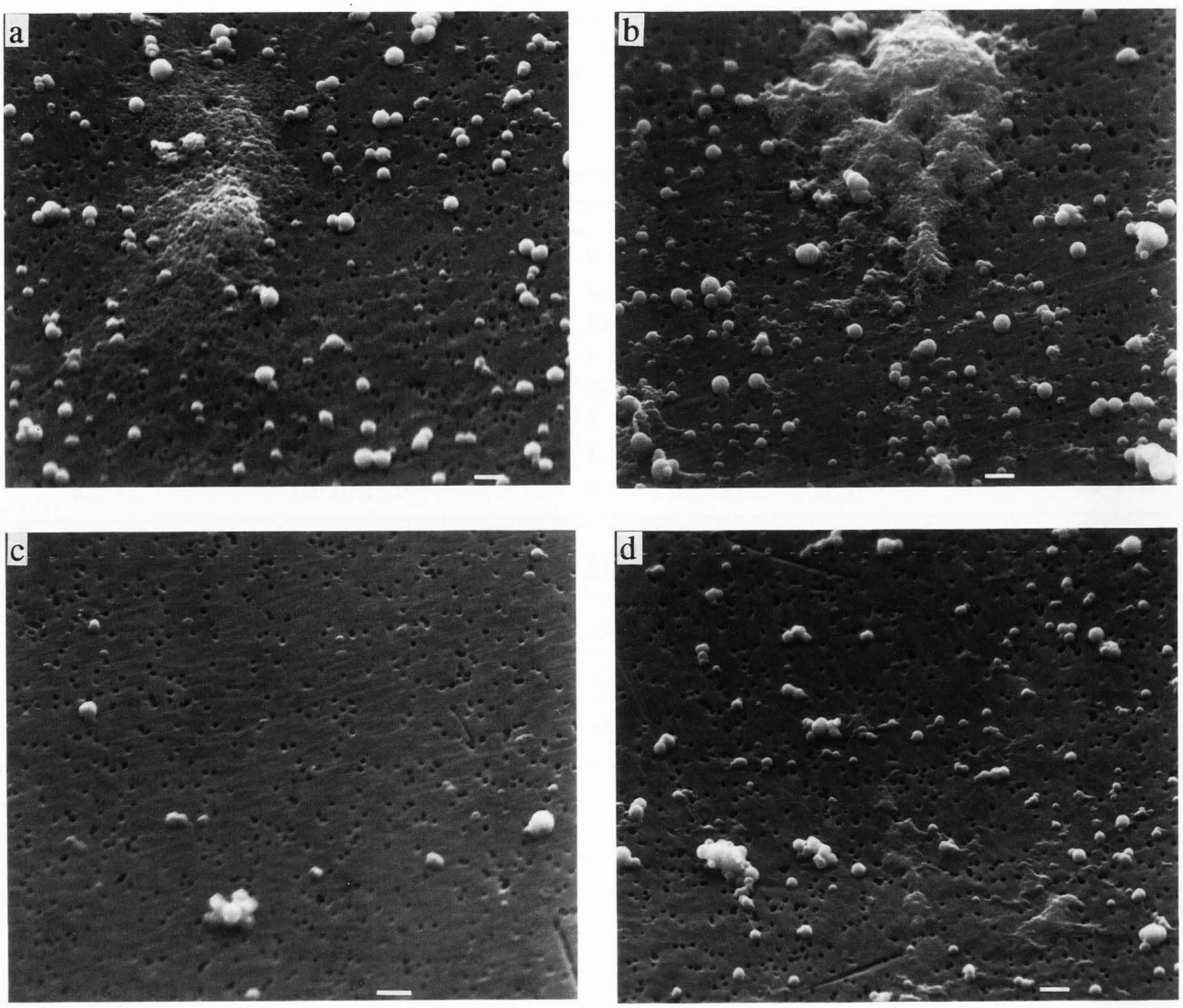

Figure 3. Scanning electron micrographs of the precipitate initially formed following addition of calcium to a PT sample without (**A**) and with (**B**) dialysed urine and a DTp sample without (**C**) and with (**D**) dialysed urine. Bars = 1 μm.

subject to a constant change during its passage through the nephron. The most pronounced alteration probably occurs during the concentration in the collecting duct and although the composition in the proximal part of the collecting duct is similar to that in the distal part of the distal tubuli, the composition in the distal part of the collecting duct will be similar to the final urine. To cover the whole range of supersaturation in the nephron, we used a composition of the CD samples approximately corresponding to that in the final urine.

Dialysed urine was added as a source of urinary macromolecules. We added dialysed urine in an amount that very roughly corresponded to the dilution in the nephron relative to the final urine. Both PT and DTp samples were used with a 10% concentration of dialysed urine, which for PT probably means a slightly higher concentration of macromolecules than that encountered physiologically, but no important differences were observed between samples with and without dialysed urine.

Although dialysed urine is an excellent source of urinary macromolecules, it had two major drawbacks in these experiments. Firstly, we paid no attention to the fact that some of the macromolecules are not normally present at all levels of the nephron (Coe *et al.*, 1991a,b; Hess, 1992), and secondly, macromolecules admixed during the storage in the bladder might influence the crystallization in a way that differs from the physiological situation (Martin *et al.*, 1985; Edyvane, 1986). This can, to some extent, be compensated for by adding pure preparations of known crystallization modifiers such as nephrocalcin (Coe *et al.*, 1991a,b), Tamm-Horsfall mucoprotein (Rose and Sulaiman, 1982; Yoshioka *et al.*, 1989; Coe *et al.*, 1991a,b; Hess, 1992), and uropontin (Hoyer, 1992), but such an approach might also be

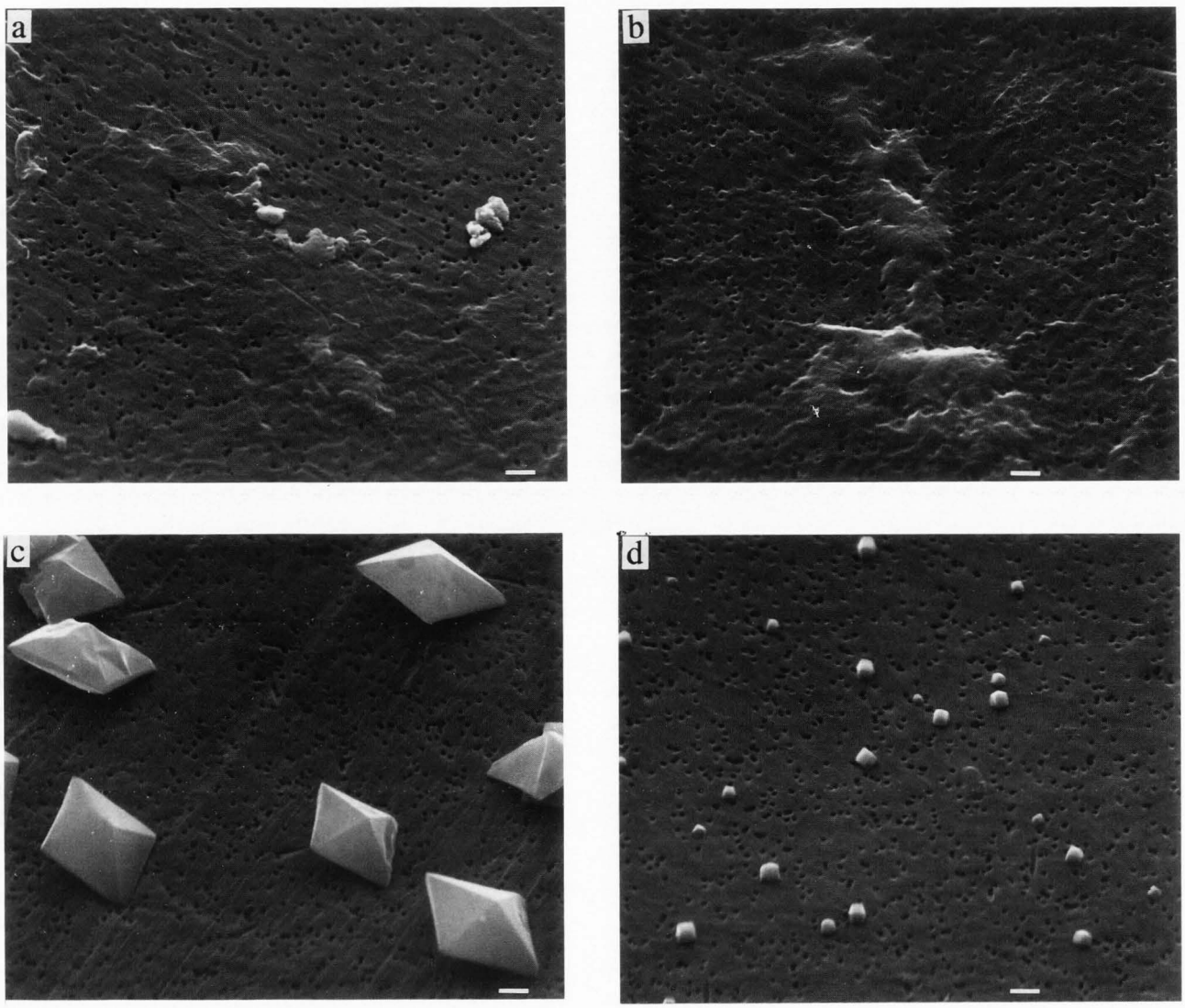

Figure 4. Scanning electron micrographs of the precipitate initially formed following addition of calcium to DTd sample without (**A**) and with (**B**) dialysed urine and a CD sample without (**C**) and with (**D**) dialysed urine. Bars = 1 μm.

insufficient because all macromolecules that affect the crystallization process are not yet identified (Atmani *et al.*, 1993). We made no attempt to assess quantitatively the effect of urinary macromolecules, and for this reason, we used a urine-pool from normal subjects that was available. Although acidification of the sediment might damage a small fraction of urinary macromolecules, such a mechanism is probably of less significance for the conclusions in this study.

The ion activity products in the various solutions were calculated by means of repeated approximation with the EQUIL2 program (Werness *et al.*, 1985). No corrections were made for the complex-formation between the several ions and urinary macromolecules (Gill *et al.*, 1977; Lian *et al.*, 1977; Morse and Resnick, 1989; Nishio *et al.*, 1990), and the ion-activity products obtained for the samples with dialysed urine might, therefore, in reality, be lower than the values presented. This effect is probably less important for the low concentrations of dialysed urine present in the PT and DT samples, but is certainly more important with the high urine concentration used in the CD samples. On the other hand, it is likely that the macromolecules affect the ion activities in a similar way before and after addition of calcium and oxalate.

The calculated estimates of AP_{CaOx}, AP_{HAP} and AP_{Bru} for the solution composition at the first appearance of crystals should not be taken for an estimate of the formation product because in our Coulter counter measurements, no particles were detected until they had increased to a diameter of at least 3.5 μm. This should be considered in view of the fact that the critical cluster size for calcium oxalate has been estimated to be 13 Å (Walton, 1967) and that of amorphous calcium phosphate

9.8 Å (Nancollas, 1982). Crystals were microscopically detected already at a calcium concentration in the CD sample of approximately 5 mmol/l which corresponds to an AP_{CaOx} of 2.13×10^{-8}. The recorded levels of AP_{HAP}, AP_{Bru} and AP_{CaOx} at the first experimental evidence of crystal formation are, however, presumably proportional to the actual formation products of the salts.

Furthermore, both calcium and oxalate were added in a standardized way during a limited period of time, during which a rapid precipitation was necessary for detection of the crystallization. It should also be emphasized that the experiments were carried out in the absence of particulate material (with a diameter above 0.22 μm) that normally might be present in the nephron urine and also without the cellular lining that probably is important for establishment of a fixed particle crystallization (Finlayson and Reid, 1978; Rushton *et al.*, 1981; Hering *et al.*, 1989; Kohri *et al.*, 1991; Mandel and Riese, 1991).

It needs to be emphasized that the reproducibility in non-seeded crystallization systems is inferior to that in seeded systems (Nancollas, 1983). For this reason, between 9 and 11 experiments were performed with each solution. Overall, the reproducibility was best in the calcium experiments. The coefficients of variation for the increment of calcium at the first appearance of crystals in the pure salt solutions varied between 6.5 and 20.5%, and in solutions with dialysed urine between 9.2 and 28.5%. The corresponding figures following addition of oxalate were between 2.3 and 23.0%, and between 4.4 and 25.1%, respectively. Although considerable, this variation is acceptable in this type of experiments.

The variability in AP_{HAP} necessary for inducing nucleation of calcium phosphate is remarkable but can possibly be explained either by the different calcium/phosphate or calcium/citrate ratios in the different solutions leading to different kinetics and different size distributions during the early phase of the crystal formation process.

Formation of crystals in PT and DTp-samples

The measurements in this investigation showed that in solutions with a composition corresponding to that in the proximal and distal tubuli, there was a greater risk of forming crystals of calcium phosphate than of calcium oxalate. This was in accordance with the supersaturation levels assumed to occur in urine at these levels of the nephron. Although an increased concentration of oxalate resulted in nucleation of calcium oxalate, the relative increment in oxalate required for crystal formation was higher than the relative increment of calcium. Although crystals of calcium oxalate have been observed at high levels of the nephron in animal experiments, this was always seen in association with a considerable load of oxalate (Kawada *et al.*, 1976; Khan *et al.*, 1979; Rushton *et al.*, 1981; Buck and Reis-Santos, 1990).

Very high relative increments were recorded for oxalate in order to induce crystallization in PT and DTp samples, and although the nucleation in reality starts at lower concentrations than those determined with this technique, it is reasonable to assume that the risk of calcium oxalate crystallization in these parts of the nephron normally is extremely low.

In a recent study based on micropuncture studies in animals, it was concluded that the loop of Henle was at particular risk for precipitation of calcium phosphate as well as of calcium carbonate (Coe and Parks, 1990; Deganello *et al.*, 1990). We have not carried out any measurements with a composition corresponding to that in the loop of Henle, but have assumed that the risk of crystallization in this part of the nephron might be between that in PT and DTp, nor did we include CO_2 in our calculations. Although this factor is fundamental for determination of the risk of calcium carbonate nucleation, its effect on calcium phosphate is negligible. It is possible, however, that the counter current system in this part of the nephron brings about changes in urine composition that are difficult to predict.

Formation of crystals in DTd and CD samples

The increment in concentrations of calcium and oxalate necessary for appearance of crystals in DTd and CD samples were lower than those in PT and DTp samples. There were several observations supporting the conclusion that the risk of calcium phosphate formation in the DTd samples was higher than that of calcium oxalate. A higher relative increment of oxalate than of calcium was required to start the crystallization. In addition, calcium phosphate was the predominant crystal phase, at least early after the first appearance of crystals. Furthermore, there was no detectable reduction in the concentration of ^{14}C-oxalate remaining in solution, when the concentration of calcium was increased beyond the point of nucleation (Figure 2).

With the composition of the CD samples used in these experiments, calcium oxalate was the predominant crystal phase observed irrespective of whether oxalate or calcium were added. The pH is certainly an important determinant of crystal formation at increased concentrations of calcium under these conditions. We used a pH of 5.75, which might be an acceptable estimate of the average pH of urine at this nephron level. The higher pH following meals, and seen in association with acidification defects, will probably increase the risk of calcium phosphate rather than calcium oxalate nucleation also in this part of the nephron.

The increments in the concentrations of oxalate necessary to get an AP_{CaOx} at the level of the formation product (around 2×10^{-8} M^2) would be approximately 0.20, 0.26, 0.55 and 0.32 mmol/l for solutions with compositions corresponding to urine in the proximal tubuli, the proximal part of the distal tubuli, the distal part of the distal tubuli, and the collecting duct. It is not likely that these concentrations will occur physiologically except in the collecting duct. Crystal nucleation on a surface with a suitable promoting lattice may, however, be possible at lower levels of supersaturation.

The inhibiting influence of macromolecules on the further development of crystals in the CD samples is evident from the great difference in MCV-quotients between pure salt solutions and salt solutions with dialysed urine. A similar effect was not observed in those samples in which calcium phosphate was the primary product of crystallization.

Some factor in the dialysed urine might act as a promoter of calcium oxalate precipitation. Such a mechanism has previously been suggested by other authors (Phaneuf-Mimeault and Tawashi, 1977; Hallson and Rose, 1979; Burns and Finlayson, 1980; Werness *et al.*, 1981; Drach *et al.*, 1982; Rose and Sulaiman, 1984; Khan *et al.*, 1988; Morse and Resnick, 1989; Hackett *et al.*, 1990; Nishio *et al.*, 1990) and is furthermore supported by the lower AP_{CaOx} required for CaOx crystal formation in the presence of dialysed urine following addition of oxalate (Table 5).

There was a higher AP_{CaOx} recorded at the first appearance of crystals in the CD sample following addition of oxalate in comparison with that following addition of calcium, despite the fact that calcium oxalate crystals were formed in both experiments. The calcium/citrate ratio might thereby have influenced the early development of crystals, that is, in the size range below 3.5 μm. It should be observed that the calcium/citrate ratio in the CD sample without urine was 1.40 at the first appearance of crystals following addition of oxalate and 3.74 following addition of calcium. A higher concentration of free citrate relative to the concentration of calcium might have increased the aggregation inhibition resulting in smaller particles and a later appearance of crystals in the size range above 3.5 μm. However, calcium oxalate was the major crystal phase, both in the calcium and the oxalate experiments.

Although calcium oxalate undoubtedly was the predominant crystal phase in CD-samples, brushite crystals were later observed following further addition of calcium . This is consistent with the supersaturation levels of brushite encountered at the first appearance of crystals in these samples. Brushite crystals apparently occurred later than calcium oxalate crystals in as much as their presence increased during storage of the sample. Brushite crystals were found in DTd samples in which AP_{Bru} was above the solubility product, but below the formation product. A proceeding crystallization during preparation of the samples for microscopy might have lowered the pH and further increased the supersaturation.

Irrespective of whether the primary nucleus is amorphous calcium phosphate or brushite, it can be stated that calcium phosphate is the crystal that most easily will form in the distal tubuli.

Possible clinical significance of the observations

On the condition that our assumptions on the urine composition in various parts of the nephron are reasonably correct, the results of the reported experiments lead to some interesting speculations on the initial steps of calcium oxalate stone formation. A high concentration of calcium at the end of the distal tubuli, in the beginning of the collecting duct, or at both places, might result in the precipitation of calcium phosphate. This precipitate could then adhere to the surface of the nephron cells. Following a slow migration to a lower level of the collecting duct, these calcium phosphate crystals might be able to induce nucleation of calcium oxalate in case of a sufficiently high supersaturation level and a pH below 6.5. The crystallization might occur either as a heterogeneous growth of calcium oxalate directly on the crystals of calcium phosphate or by a nucleation of calcium oxalate induced by the macromolecules adsorbed to this material. The simultaneous nucleation of brushite in the collecting duct urine might add further calcium phosphate to the precipitate and also provide additional promoting nuclei for calcium oxalate crystallization. There is so far insufficient evidence for proof of such a hypothesis, but the results obtained in this study clearly show that the complexity of calcium oxalate stone formation most certainly cannot be disentangled by only regarding the crystallization properties in samples of whole urine.

Acknowledgement

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Discussion with Reviewers

W.G. Robertson: Based on the authors' data, it would seem unlikely that hypercalciuria per se can be considered as a major cause of crystal formation in most parts of the nephron. What is the importance of hypercalciuria in calcium stone disease?

Authors: The increase in calcium concentrations necessary for crystal formation in PT and DTp samples indicates that a crystallization of calcium phosphate induced by hypercalciuria certainly is low at these levels. Also in DTd and CD samples, it was necessary to increase markedly the concentration of calcium in order to record

crystal formation, although the relative increment at these concentration levels was much lower than in DTp-samples. No conclusions can be drawn, however, on the calcium concentration required for nucleation, because our method did not detect crystals until they had achieved a diameter of at least 3.5 μm. Therefore, the calcium concentration recorded for the first appearance of crystals is much higher than that necessary for nucleation. The methods for assessing crystallization are not sensitive enough to decide on the role of hypercalciuria. The results, however, demonstrate that if an increased excretion of calcium is of importance at these levels of the nephron, then calcium phosphate is the most likely product of crystallization in the distal tubuli. The risk of calcium oxalate crystallization increases as the urine moves further down the nephron in a direction towards the distal part of the collecting duct.

W. G. Robertson: The activity products necessary to produce crystallization of both calcium oxalate and calcium phosphate seem much higher than anticipated from some other studies. What explanation do the authors have for this observation?

Authors: The explanation for this phenomenon should also be sought in the methodology. Both calcium and oxalate were added to the solutions in a standardized way to facilitate a comparison between different samples. In addition to the fact that no crystals with a diameter below 3.5 μm were recorded with the Coulter counter technique, the samples were supersaturated to a higher level than that required for the formation of a critical nucleus of approximately 10 to 13 Å. The recorded ion-activity products are not estimates of the formation products but they will hopefully reflect a process that actually starts at much lower level of supersaturation. It is important to emphasize, however, that the concentration of calcium and oxalate necessary for forming a 3.5 μm crystal is highly dependent upon the kinetics of crystal growth and crystal aggregation during the early phase of crystallization. Based on the preliminary data reported above, studies are now in progress aiming at a more accurate assessment of the risk of calcium oxalate and calcium phosphate in solutions with a composition corresponding to that in the distal part of the distal tubuli and in different parts of the collecting duct. Only following such experiments will it be possible to draw any conclusions on the clinical significance of the risk of calcium phosphate and calcium oxalate nucleation at these levels of the nephron.